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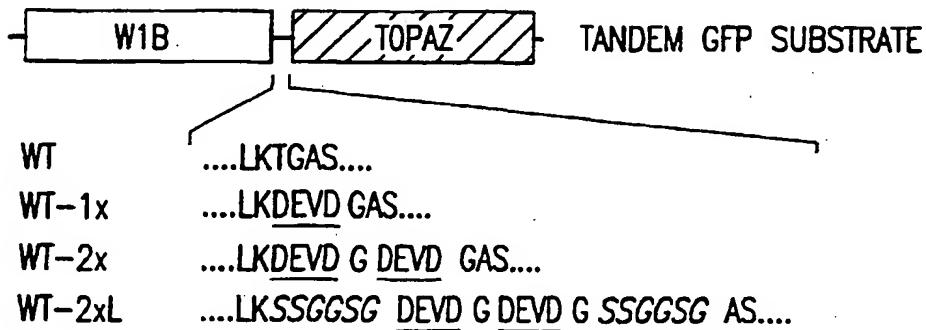
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(54) Title: ASSAYS FOR CASPASE ACTIVITY USING GREEN FLUORESCENT PROTEINS



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(57) Abstract: Provided are substrates and assays for the identification of caspase activators and inhibitors. The substrates are fusion proteins comprising two green fluorescent proteins (GFPs) with a linker peptide comprising at least one caspase cleavage site. The intact fusion protein exhibits fluorescent resonance energy transfer (FRET) between the GFPs. Following caspase cleavage of the linker peptide, the two GFPs become separated and FRET is diminished.

## TITLE OF THE INVENTION

ASSAYS FOR CASPASE ACTIVITY USING GREEN FLUORESCENT PROTEINS

## 5 CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

## STATEMENT REGARDING FEDERALLY-SPONSORED R&amp;D

Not applicable.

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## REFERENCE TO MICROFICHE APPENDIX

Not applicable.

## FIELD OF THE INVENTION

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The present invention is directed to methods for determining the activity of caspases. The methods utilize fluorescent resonance energy transfer between two green fluorescent proteins linked as a fusion protein having a caspase cleavage site between them. The methods can be used to identify substances that are activators or inhibitors of caspase activity either in living cells or *in vitro*.

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## BACKGROUND OF THE INVENTION

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Apoptosis is characterized by distinct series of morphological and biochemical changes that inevitably result in the death of a cell (Kerr et al., 1972, Br. J. Cancer 26:239-257; Vaux et al., 1994, Cell 76:777-779; Steller, 1995, Science 267:1445-1449). Apoptosis occurs during developmental morphogenesis, in the removal of expended, unnecessary or irreparably damaged cells, and in response to pathogenic infections (Kerr et al., 1972, Br. J. Cancer 26:239-257; McConkey et al., 1996, Mol. Aspects Med. 17:1-110; Steller, 1995, Science 267:1445-1449; Thompson, 1995, Science 267:1456-1462; Uren & Vaux, 1996, Pharmacol. Ther.

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72:37-50). Apoptosis proceeds through a highly regulated series of biochemical events and many of the components of the cell death pathway have been identified. Among the key biochemical mediators of apoptosis are a group of proteolytic enzymes known as the caspases, which serve to disrupt normal cellular homeostasis by cleaving specific target proteins with either structural, regulatory or housekeeping

functions (Thornberry & Lazebnik, 1998, *Science* 281:1312-1316; Thornberry, 1997, *Br. Med. Bull.* 53:478-490; Nicholson & Thornberry, 1997, *Trends Biochem. Sci.* 22:299-306). This results in the cessation of normal cellular functions, the dismantling of the genome and structural constituents of the cell, and the packaging of 5 cellular components into apoptotic corpses for engulfment by other cells (Nicholson & Thornberry, 1997, *Trends Biochem. Sci.* 22:2199-306; Rosen & Casciola-Rosen, 1997, *J. Cell. Biochem.* 64:50-54).

The caspases comprise a large family of proteins that can be distinguished according to their substrate specificities (Thornberry, 1997, *J. Biol. Chem.* 272:17907-17911). Twelve caspases have so far been identified in human 10 cells. All caspases are synthesized as dormant proenzymes and can be rapidly activated through proteolytic maturation (Thornberry & Lazebnik, 1998, *Science* 281:1312-1316; Thornberry, 1997, *Br. Med. Bull.* 53:478-490). Members of the caspase family can be divided into three functional subgroups based on their substrate 15 specificities which have been defined by a positional-scanning combinatorial substrate approach (Rano et al., 1997, *Chem. Biol.* 4:149-155; Thornberry et al., 1997, *J. Biol. Chem.* 272:17907-17911). The principal effectors of apoptosis (group II caspases, which include caspases-2, -3 and -7 as well as *C. elegans* CED-3) have specificity for [P<sub>4</sub>]DExD[P<sub>1</sub>], a motif found at the cleavage site of most proteins 20 known to be cleaved during apoptosis (Nicholson and Thornberry, 1997, *Trends Biochem. Sci.* 22:299-306). On the other hand, the specificity of group III caspases (caspases-6, -8, -9 and -10, as well as CTL-derived granzyme B) is [P<sub>4</sub>](I,V,L)ExD[P<sub>1</sub>] which corresponds to the activation site at the junction between 25 the large and small subunits of other caspase proenzymes, including group II (effector) family members. This and other evidence indicates that group III caspases function as upstream activators of group II caspases in a proteolytic cascade that amplifies the death signal.

Given their central role as death effector molecules, a great deal of 30 interest has recently focused on the caspases as therapeutic targets for various disease processes (e.g., acute neurodegeneration, cardiac ischemia, hepatic failure) (Nicholson, 1996, *Nat. Biotechnol.* 14:297-301). Unfortunately, methods for monitoring caspase activity, at least in the context of cell-based systems, are primarily limited to analyzing downstream biochemical events (e.g., DNA fragmentation, membrane remodeling) that are often several steps removed from the proteolysis

caused by active caspases (Darzynkiewicz & Traganos, 1998, *Adv. Biochem. Eng. Biotechnol.* 62:33-73). In addition, sensitive substrates which are cell permeable and specific for different caspases subgroups have yet to be developed. Thus, there is a need for substances that can be used to monitor caspase activity that are sensitive, 5 specific for particular caspases. provide readouts in real time, and can act intracellularly.

GFP-based reporter strategies have been used in numerous cellular systems to monitor transcriptional activation, cellular localization and protein trafficking (Misteli et al., 1997, *Nat. Biotechnol.* 15:961-964; Tsien, 1998, *Annu. Rev. Biochem.* 67:509-544). Their utility as *in vitro* substrates for protease assays have also been demonstrated (Pollok & Heim, 1999, *Trends Cell Biol.* 9:57-60; Mitra et al., 1996, *Gene* 173:13-17; Heim et al., 1996, *Curr. Biol.* 6:178-182). Xu et al., 1998, *Nucl. Acids Res.* 26:2034-2035 (Xu) disclosed the use of a fusion protein comprising a blue fluorescent protein and a green fluorescent protein linked by an 18 10 amino acid peptide containing a caspase-3 cleavage site. This fusion protein was used to monitor the activation of caspase activity by the use of fluorescence activated cell sorting (FACS) as well as by detection of fragments of the fusion protein by Western 15 blot. Xu did not disclose methods of high-throughput screening for identifying inhibitors of caspases.

Heim & Tsien. 1996, *Current Biology* 6:178-182 disclosed the use of a fusion protein comprising two GFPs separated by a linker of 25 amino acids that could be cleaved by trypsin or enterokinase. International Patent Publication WO 97/28261 disclosed the use of fusion proteins comprising two GFPs separated by a linker that can be cleaved by a protease. Mitra et al., 1996, *Gene* 173:13-17 disclosed 20 the use of a fusion protein comprising two GFPs separated by a linker of 20 amino acids that could be cleaved by Factor Xa. 25

## SUMMARY OF THE INVENTION

The present invention is directed to methods of monitoring caspase activity by utilizing fluorescent resonance energy transfer between two green fluorescent proteins linked as a fusion protein having a caspase cleavage site between them. The methods are fast, sensitive, and generally act at the level of caspase activity, *i.e.*, proteolysis of caspase substrates, rather than at the level of downstream events. The methods can be practiced *in vitro*, *e.g.*, using cell-free extracts or purified

components, or the methods can be practiced *in vivo*, *i.e.*, in living cells. The methods can be adapted to provide assays suitable for the identification of activators as well as inhibitors of caspase activity.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-B shows fluorescence resonance energy transfer between mutant GFP variants in a recombinant group II caspase substrate. Figure 1A: Upon excitation of the donor GFP (W1B) at 434 nm, its emitted energy (476 nm) is transferred to the acceptor GFP (TOPAZ) and is re-emitted at 527 nm. This energy transfer is dependent on the close physical proximity of the two GFP proteins.

10 Caspase-mediated cleavage of the linker between the GFPs abrogates energy transfer through separation of the GFP molecules. Figure 1B: Outlined below the schematic of the tandem GFP protein is the primary amino acid sequence of the linker for each of the fusion proteins constructed (WT, WT-1x, WT-2x, and WT-2xL). Note that the

15 WT fusion contains a short six amino acid linker lacking a caspase tetrapeptide cleavage motif. The WT-1x and WT-2x proteins contain a single and two tandem group II caspase cleavage motifs (DEVD (SEQ.ID.NO.:9)) within the linker, respectively. WT-2xL contains two tandem DEVD (SEQ.ID.NO.:9) cleavage motifs flanked by six amino acid SG repeats designed to confer flexibility and accessibility

20 to the linker. The amino acid sequence shown for the WT linker is SEQ.ID.NO.:1; The amino acid sequence shown for the WT-1x linker is SEQ.ID.NO.:2; The amino acid sequence shown for the WT-2x linker is SEQ.ID.NO.:3; The amino acid sequence shown for the WT-2xL linker is SEQ.ID.NO.:4.

Figure 2A-B shows that FRET efficiency is a function of the linker length. Lysates from HeLa cells transfected transiently with constructs encoding the WT, WT-1x, WT-2x, or WT-2xL fusion proteins were assayed for fluorescence.

Figure 2A shows the emission scan (450-600 nm) of the lysates following excitation at 434 nm. Figure 2B shows measurement of FRET by ratiometric analysis (expressed as the ratio of the fluorescence emitted by the acceptor (TOPAZ, 527 nm) divided by the fluorescence emitted by the donor (W1B, 476 nm) (n=2)).

Figure 3 shows that tandem GFP substrates containing multiple DEVD (SEQ.ID.NO.:9) motifs are cleaved more efficiently by caspase-3 than substrates containing a single DEVD (SEQ.ID.NO.:9) motif. *In vitro* translated and [<sup>35</sup>S]methionine-labeled WT-1x and WT-2x proteins were incubated with varying

concentrations of purified recombinant caspase-3 for 1 hr at 37°C. Cleaved proteins were resolved by SDS polyacrylamide gel electrophoresis and quantitated by autoradiography and scanning densitometry of the gels. The reactions were carried out using concentrations of substrate well below Km, where the appearance of the product is a first-order process. Values for  $k_{cat}/K_m$  were calculated from the relationship  $S_t/S_0 = e^{-k_{obs} \cdot t}$  where  $S_t$  is the concentration of the substrate remaining at time  $t$ ,  $S_0$  is the initial substrate concentration, and  $k_{obs} = k_{cat} \cdot [enzyme]/K_m$ .

Figure 4A-B shows that apoptosis results in cleavage of DEVD (SEQ.ID.NO.:9)-containing tandem GFP substrates. Figure 4A shows Western blot analysis of the tandem GFP substrates in lysates prepared from HeLa cells transfected with the WT, WT-1x, or WT-2x constructs. Apoptosis was induced by treatment of the cells for 4 hrs with 1  $\mu$ M staurosporine. Figure 4B shows FRET measurements from cells treated with staurosporine in the presence (+) or absence (-) of a pan caspase inhibitor (zVAD-fmk). Note that FRET is diminished in apoptotic extracts containing caspase-3 sensitive tandem GFP substrates only.

Figure 5 shows the direct fluorometric measurement of caspase inhibition in intact cells. HeLa cells (clone HETON-WT-1x-57) overexpressing the WT-1x tandem GFP substrate were treated simultaneously with staurosporine to induce apoptosis and varying concentrations of zVAD-fmk for 4 hrs. Cells were assayed for FRET (530 nm./490 nm.) after exchanging the media for PBS. The 0.01  $\mu$ M and 10,000  $\mu$ M titration points represent values for the maximum (DMSO vehicle alone) and minimum (staurosporine alone) FRET ratios obtained, respectively.

Figure 6A-B shows high-throughput screening of caspase inhibitors in multiwell microtiter plates containing either single compounds (Figure 6A) or drug mixtures (10 compounds/well) (Figure 6B). Duplicate 96-well plates were co-treated with staurosporine and drug(s) for four hours. Three concentrations of zVAD-fmk (2.5  $\mu$ M, 25  $\mu$ M, and 250  $\mu$ M) were randomly added to two wells in each plate. The experiment was performed blindly, such that the well location of zVAD-fmk was not revealed until the data were processed. The black and gray bars indicate the position of the wells containing 250  $\mu$ M and 25  $\mu$ M zVAD-fmk, respectively. False-positive hits are indicated by the striped bars. Column 1 (row A-H) represents uninduced nonapoptotic control wells.

Figure 7A-B shows the nucleotide (Figure 7A, SEQ.ID.NO.:5) and the amino acid (Figure 7B, SEQ.ID.NO.:6) sequences of wild-type green fluorescent protein.

## 5 DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

A "green fluorescent protein (GFP)" is a fluorescent protein in which any 150 contiguous amino acids have an amino acid sequence identity of at least 85% to a contiguous stretch of the amino acid sequence of wild-type GFP (SEQ.ID.NO.:6).

10 An "ultra-bright green fluorescent protein (GFP)" is a GFP that has been engineered to contain amino acid changes from wild-type GFP that result in enhanced emission properties compared to wild-type GFP. Ultra-bright GFPs do not have the change Y66H. Ultra-bright GFPs have a fluorescence quantum yield of at least 0.39. Examples of ultra-bright GFPs are W1B; TOPAZ; 10C; 10C Q69K; W7; 15 W1C; wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, S72A, and T203F; and wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, S72A, and T203H.

20 "High-throughput screening" refers to methods of identifying activators or inhibitors of a biological activity, *e.g.*, the activity of a caspase, in which a large number of substances suspected of having a desired property, *e.g.*, being an activator or an inhibitor of a caspase, are tested to determine if in fact such substances have such a property. High-throughput screening involves the testing of at least 5,000, preferably at least 10,000, and even more preferably at least 100,000 substances per 24 hour period to determine if any of the substances have the desired property. The methods of high-throughput screening are carried out in volumes of less than 250  $\mu$ l, preferably less than 25  $\mu$ l, and even more preferably less than 10  $\mu$ l 25 in microtiter-like plates having at least 96 wells, preferably at least 384 wells, and even more preferably at least 1,536 wells per plate.

30 "Ratiometric analysis" refers to measurement of fluorescence resonance energy transfer (FRET) by determining the ratio of the fluorescence emitted by an acceptor green fluorescent protein (*e.g.*, TOPAZ, 527 nm) divided by the fluorescence emitted by a donor green fluorescent protein (*e.g.*, W1B, 476 nm) after excitation of the donor green fluorescent protein.

"Fusion protein" refers to a polypeptide comprising a sequence of amino acids in which two green fluorescent proteins (GFPs) are joined by a linker that

consists of a short stretch of amino acids where the linker comprises at least one caspase cleavage site. One GFP is a donor GFP and the other GFP is an acceptor GFP. The two GFPs are different GFPs such that fluorescence resonance energy transfer (FRET) can occur from the donor GFP to the acceptor GFP when the linker is intact but, upon cleavage of the linker, FRET is abolished or greatly diminished.

5 "Caspase inhibitor" refers to a substance that decreases the proteolytic activity of a caspase protein. A caspase inhibitor has no necessary effect on the amount of caspase protein produced by a cell. Nor does a caspase inhibitor necessarily act at the level of proteolysis by a caspase. A caspase inhibitor may 10 decrease the proteolytic activity of a caspase protein by acting at the level of upstream events that result in the activation of the caspase. For example, a caspase inhibitor may act to prevent the release of cytochrome c from mitochondria, thus preventing the activation of a caspase. The methods of the present invention provide means for identifying caspase inhibitors. Once identified, such inhibitors can be further 15 characterized with respect to the level at which they act. One possibility would be to test the identified inhibitor in an *in vitro*, *i.e.*, cell-free system comprising purified caspase and GFP fusion protein substrate. If the inhibitor is able to prevent the loss of FRET from the fusion protein under such conditions, then the inhibitor is acting at the level of proteolysis by the caspase. Other such secondary tests will be evident to 20 those of skill in the art.

"Caspase activator" refers to a substance that increases the proteolytic activity of a caspase protein. A caspase activator has no necessary effect on the amount of caspase protein produced by a cell. Nor does a caspase activator necessarily act at the level of proteolysis by a caspase. A caspase activator may 25 increase the proteolytic activity of a caspase protein by acting at the level of upstream events that result in the activation of the caspase. As for caspase inhibitors, the level at which a caspase activator acts can be determined by suitable *in vitro* assays.

The present invention makes use of fluorescence resonance energy transfer (FRET). FRET is a process in which energy is transferred in a non-radiative manner from an excited donor fluorescent reagent to an acceptor fluorescent reagent by means of intermolecular long-range dipole-dipole coupling. FRET typically occurs over distances of about 10Å to 100Å and requires that the emission spectrum of the donor reagent and the absorbance spectrum of the acceptor reagent overlap adequately and that the quantum yield of the donor and the absorption coefficient of

the acceptor be sufficiently high. In addition, the transition dipoles of the donor and acceptor fluorescent reagents must be properly oriented relative to one another. For reviews of FRET and its applications to biological systems, see, e.g., Clegg, 1995, Current Opinions in Biotechnology 6:103-110; Wu & Bränd, 1994, Anal. Biochem. 218:1-13.

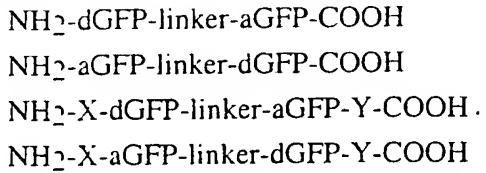
5 The present invention is directed to fusion proteins comprising two green fluorescent proteins (GFPs) linked by a peptide comprising at least one caspase cleavage site and uses of the fusion proteins in methods of identifying substances that are activators or inhibitors of caspase activity either in cells or *in vitro*. The fusion 10 proteins comprise a donor GFP and an acceptor GFP. FRET occurs between the donor GFP and the acceptor GFP when the donor GFP is excited and when the linker between the donor GFP and the acceptor GFP is intact. Caspase cleavage of the linker separates the donor and acceptor GFPs, thus abolishing FRET between them.

15 The fusion proteins preferably are derived from GFPs having altered, enhanced spectral properties as compared with wild-type GFPs. Such GFPs are known in the art (see, e.g., U.S. Patent No. 5,625,048; International Patent Publication WO 97/28261; International Patent Publication WO 96/23810). Especially preferred are the GFPs W1B and TOPAZ, available commercially from Aurora Biosciences Corp., San Diego, CA. W1B contains the following changes from the wild-type GFP 20 sequence: F64L, S65T, Y66W, N146I, M153T, and V163A (see Table 1, page 519, of Tsien, 1998, Ann. Rev. Biochem. 67:509-544). TOPAZ contains the following changes from the wild-type GFP sequence: S65G, V68L, S72A, and T203Y (see Table 1, page 519, of Tsien, 1998, Ann. Rev. Biochem. 67:509-544). Wild-type 25 nucleotide and amino acid sequences of GFP are shown in Figure 1 and SEQ.ID.NO.: 1 of International Patent Publication WO 97/28261; in Figure 1 of Tsien, 1998, Ann. Rev. Biochem. 67:509-544; in Prasher et al., 1992, Gene 111:229-233; and in Figure 7A-B of this application.

30 While a wide variety of GFPs are suitable for use in the present invention, the choice of GFPs to use in the fusion protein should be guided by certain general considerations. The excitation spectra of the donor and acceptor GFP should overlap as little as possible. This will allow the donor to be excited without directly exciting the acceptor. The emission spectrum of the donor GFP should overlap as much as possible with the excitation spectrum of the acceptor GFP. The emission spectra of the donor and acceptor should overlap as little as possible. The quantum

yield of the donor and the extinction coefficient of the acceptor should be as high as possible.

Either the donor or the acceptor GFP may be at the amino or the carboxy terminal portion of the fusion protein. Also, additional peptide sequences 5 may be present at the amino or carboxy terminal ends of the fusion proteins. In other words, suitable fusion proteins of the present invention may be represented by the following schematics:



where dGFP = donor GFP; aGFP = acceptor GFP; X = any amino acid or amino acid sequence; Y = any amino acid or amino acid sequence; and where X and Y can be the same or different.

15 The intact fusion proteins preferably are capable of FRET to the extent that ratiometric analysis will show a FRET ratio of at least 1.5, preferably at least 2.0. That is, the ratio of the fluorescence emitted by the acceptor GFP divided by the fluorescence emitted by the donor GFP when the donor GFP is excited will be at least 1.5 and preferably at least 2.0. Such ratios are especially suitable when using the 20 methods of the present invention as a high-throughput screening tool since such ratios permit sensitive detection of caspase activation and allow for the exploitation of the advantages of ratiometric analysis, as described herein.

25 The linker is a peptide, *i.e.*, a short stretch of amino acids, of length between about 4 and 50 amino acids. Preferred are linkers of length 10 amino acids or fewer, including the caspase cleavage site. The linker comprises a single caspase cleavage site or multiple caspase cleavage sites that may be the same or different. Generally, the caspase cleavage site is chosen so that it is recognized by a single caspase. However, for some purposes, it may be preferable to use cleavage sites that 30 are recognized by more than one caspase. It may even be advantageous to employ a linker that comprises more than a single caspase cleavage site where the cleavage sites are recognized by different caspases. Such linkers are useful when it is desired to assay for activators or inhibitors of more than a single caspase.

Analysis of caspase activity in intact cells has been generally limited to the measurement of end-point biochemical and morphological markers of apoptosis

such as DNA fragmentation, membrane remodeling, chromatin condensation, cell shrinkage, changes in potential across the mitochondrial membrane, and detection of the products produced by the cleavage of caspase substrates. Such markers suffer from the disadvantages that they are downstream effects not measured in real time (i.e., as caspase cleavage is actually taking place), they generally require the harvesting of cells, and often require specialized reagents (e.g., antibodies to caspase substrates).

Methods of identifying activators and inhibitors of caspases in living cells are provided by the present invention. These methods generally involve the use of cells that have been engineered to express a fusion protein comprising two green fluorescent proteins (GFPs) linked by a peptide comprising at least one caspase cleavage site. Generally, such cells are produced by transfecting cells with expression vectors comprising DNA encoding the fusion proteins. The amount of fluorescence resonance energy transfer (FRET) in the engineered cells from the donor GFP in the fusion protein to the acceptor GFP is determined. Preferably, this determination is done by means of ratiometric analysis. In methods of identifying activators of caspases, the engineered cells are exposed to a substance that is suspected of being an activator of a caspase. If the substance is actually an activator, this results in an increase in caspase activity within the cells, leading to cleavage of the linker of the fusion protein. The two GFPs making up the fusion protein are then free to drift apart, decreasing the amount of FRET between them. This decrease in FRET is measured, preferably by ratiometric analysis. Methods of identifying inhibitors of caspases are similar, except that while the cells that are treated to induce caspase activity and/or apoptosis, they are also exposed to a substance that is suspected of being an inhibitor of caspase activity. If the substance actually is an inhibitor, cleavage of the fusion protein will be reduced or eliminated, leading to less of a decrease in FRET than is seen in the case where the cells are not exposed to the substance.

Accordingly, the present invention includes a method of identifying a caspase activator comprising:

(a) providing cells comprising a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site;

(b) measuring the amount of fluorescence resonance energy transfer (FRET) in the cells in the absence of a substance suspected of being an activator of the expression of a caspase;

5 (c) exposing the cells to the substance suspected of being an activator of the expression of the caspase;

(d) measuring the amount of FRET in the cells in the presence of the substance suspected of being an activator of the expression of the caspase;

where if the amount of FRET measured in step (b) is greater than the amount of FRET measured in step (d), then the substance is a caspase activator.

10 The present invention also includes a method of identifying a caspase inhibitor comprising:

(a) providing cells comprising a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site;

15 (b) treating the cells in order to activate a caspase;

(c) measuring the amount of fluorescence resonance energy transfer (FRET) in the treated cells in the absence of a substance suspected of being a caspase inhibitor;

20 (d) exposing the treated cells to the substance suspected of being a caspase inhibitor:

(e) measuring the amount of FRET in the treated cells that have been exposed to the substance suspected of being a caspase inhibitor;

where if the amount of FRET measured in step (e) is greater than the amount of FRET measured in step (c), then the substance is a caspase inhibitor.

25 In particular embodiments of the above-described methods, the cells are eukaryotic cells. In another embodiments, the cells are mammalian cells or are yeast cells. In other embodiments, the cells are L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651),  
30 CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), PC12 cells, *C. elegans* cells, zebrafish cells, *Xenopus* oocytes, *Xenopus melanophores*, or *Saccharomyces cerevisiae* cells.

In particular embodiments of the above-described methods, the cells are cells within a whole, living organism such as, *e.g.*, the nematode *C. elegans* or the zebrafish. The fusion proteins are introduced into the organism by methods well known in the art. An advantage of using *C. elegans* and zebrafish is that these 5 organisms are translucent, making observation of FRET in a living animal possible. In this way, the activity of caspase activators and inhibitors can be monitored in real time in a whole organism. Alternatively, cells can be prepared from these organisms that comprise the fusion protein and such cells can be used to practice the methods of the invention.

10 In particular embodiments, the present invention makes use of transgenic animals, *e.g.*, transgenic mice, in which at least some cells express a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site. Such transgenic animals are useful in understanding the role of caspases in conditions such as, *e.g.*, cardiovascular 15 diseases, neurodegenerative diseases, traumatic or septic shock, and stroke. For example, a transgenic mouse expressing the fusion protein in its neural tissue is a useful model for assessing the effects of caspase inhibitors on a chronic or acute insult, *e.g.*, neurodegenerative diseases, brain trauma, or stroke. In this model as applied to stroke, one would induce stroke in the transgenic mouse in the presence 20 and in the absence of a caspase inhibitor. The brain tissue of the mouse would then be sectioned and imaged by microscopy to determine if the caspase inhibitor is reaching particular areas of the brain and is inhibiting a caspase in those areas. Analysis of the effects of the inhibitor combined with analysis of stroke damage can 25 determine whether there is a correlation between caspase inhibition and protection from stroke damage. The present invention can therefore be used to evaluate the ability of caspase inhibitors to protect brain tissue from damage due to stroke.

In particular embodiments of the above-described methods, the cells are adherent cells rather than suspension cells. In particular embodiments, the methods are practiced in the wells of a microtiter-like plate and the cells are not 30 harvested or detached from the wells, *i.e.*, the cells remain attached to the bottoms of the wells throughout the practice of the method.

In certain embodiments of the above-described methods, an expression vector encoding the fusion protein is transfected into the cells. Such an expression vector can be made by well-known recombinant DNA methods such as those

described in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press; or PCR Primer, A Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press. One method would involve starting with a 5 standard expression vector. DNA sequences encoding the donor and acceptor GFPs are cloned into the polylinker site of the standard expression vector, preferably after PCR of the GFPs so that their sequences are flanked by restriction enzyme cleavage sites. After the GFPs have been cloned into the expression vector, there will be a restriction site or sites between the GFP sequences into which the linker can be 10 cloned. The linker sequence is chosen so that the donor GFP, acceptor GFP, and linker are all in the same reading frame in the final expression construct. Examples of the construction of a fusion protein comprising two GFPs separated by a 20 amino acid linker having a protease site (in this case a Factor Xa site) are described in Mitra et al., 1996, Gene 173:13-17. International Patent Publication WO 97/28261, at 15 Example 1, beginning at page 40, discloses methods for constructing GFP fusion proteins containing linkers having a variety of protease cleavage sites. The general procedures disclosed in these references can be used as guidelines for constructing the caspase-specific fusion proteins of the present invention.

Transfection can be accomplished by any of the methods known in the 20 art, *e.g.*, calcium phosphate or calcium chloride mediated transfection, electroporation, infection with a retroviral vector. The cells can be transiently or stably transfected.

The present invention includes DNA encoding fusion proteins comprising two green fluorescent proteins (GFPs) linked by a peptide comprising at 25 least one caspase cleavage site. The present invention also includes expression vectors comprising DNA encoding fusion proteins comprising two green fluorescent proteins (GFPs) linked by a peptide comprising at least one caspase cleavage site as well as host cells comprising these expression vectors.

A variety of standard expression vectors can be used to produce the 30 expression vector encoding the fusion protein. Commercially available expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene, La Jolla, CA), pSG5 (Stratagene, La Jolla, CA), pcDNA1 and pcDNA1amp, pcDNA3, pcDNA3.1, pCR3.1 (Innitrogen, Palo Alto, CA), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt

(ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), and pIRES1neo (Clontech, Palo Alto, CA). The choice of vector will depend upon cell type used, level of expression desired, and the like. When expressing GFPs in mammalian cells, it may be advantageous to construct versions of the GFPs having 5 altered codons that conform to those codons preferred by mammalian cells (Zolotukhin et al., J. Virol. 1996, 70:4646-46754; Yang et al., 1996, Nucl. Acids Res. 24:4592-4593). Another way of improving GFP expression in mammalian cells is to provide an optimal ribosome binding site by the use of an additional codon immediately after the starting methionine (Crameri et al., 1996, Nature Biotechnology 10 14:315-319).

For certain embodiments, it may be advantageous to use an expression vector in which the GFP fusion protein can be cloned under the control of an inducible promoter such as, e.g., promoters induced by dexamethasone, metallothionein, or tetracycline and related analogs such as doxycycline. This can 15 circumvent any toxicity problems that might arise as a result of the unregulated high level expression of GFPs. A suitable vector containing an inducible promoter is the pTRE vector from Clontech, Palo Alto, CA; this vector contains a tetracycline inducible promoter. Other such vectors are well-known in the art and many are available commercially.

20 In certain embodiments, the donor and acceptor GFPs are selected from the group consisting of:

W1B, wild-type GFP (SEQ.ID.NO.:6) having the changes F64L, S65T, Y66W, N146I, M153T, and V163A;

TOPAZ, wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, 25 V68L, S72A, and T203Y;

wild-type GFP (SEQ.ID.NO.:6);

wild-type GFP (SEQ.ID.NO.:6) having the change Q80R (Chalfie et al., 1994, Science 263:802-805);

wild-type GFP (SEQ.ID.NO.:6) having the change S65T;

30 wild-type GFP (SEQ.ID.NO.:6) having the change Y66W;

wild-type GFP (SEQ.ID.NO.:6) having the change Y66F;

wild-type GFP (SEQ.ID.NO.:6) having the changes F64L, S65T, and V163A;

wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, S72A, and T203F;

wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, S72A, and T203H;

5 Cycle 3. wild-type GFP (SEQ.ID.NO.:6) having the changes F99S, M153T, and V163A;

EGFP. wild-type GFP (SEQ.ID.NO.:6) having the changes F64L and S65T;

10 Emerald. wild-type GFP (SEQ.ID.NO.:6) having the changes S65T, S72A, N149K, M153T, and I167T;

H9. wild-type GFP (SEQ.ID.NO.:6) having the changes S202F and T203I;

H9-40. wild-type GFP (SEQ.ID.NO.:6) having the changes T203I, S72A, and Y145F;

15 10C Q69K, wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, V68L, Q69K, S72A, and T203Y;

10C. wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, V68L, S72A, and T203Y;

W7. wild-type GFP (SEQ.ID.NO.:6) having the changes Y66W,

20 N146I, M153T, and V163A;

W1C. wild-type GFP (SEQ.ID.NO.:6) having the changes S65A, Y66W, S72A, N146I, M153T, and V163A;

BFP. wild-type GFP (SEQ.ID.NO.:6) having the change Y66H;

25 P4-3. wild-type GFP (SEQ.ID.NO.:6) having the change Y66H and Y145F; and

EBFP. wild-type GFP (SEQ.ID.NO.:6) having the change F64L, Y66H, and Y145F.

A variety of suitable donor and acceptor GFPs and their spectral characteristics are disclosed in Table 1, page 519, of Tsien, 1998, Ann. Rev.

30 Biochem. 67:509-544.

In certain embodiments of the methods of identifying a caspase activator or inhibitor, the step of treating the cells in order to activate a caspase is performed by: viral infection; growth factor withdrawal; anticancer agents; exposure to ultraviolet radiation; treatment with staurosporine, actinomycin D, etoposide,

glucocorticoids, camptothecin, thapsigargin, cyclohexamide, rapamycin, ceramide, glutamate, N-methyl-D-aspartate, kainic acid, lectins, tributyltin, vincristine, vinblastine, cisplatin, pentoxifyllin, cytotrienin A, 6-hydroxydopamine, 1,10-phenanthroline, nitric oxide, or sodium butyrate; enforced c-myc expression; or the 5 stimulation of tumor necrosis factor receptor (TNFR) family members (*e.g.*, Fas/Fas ligand interaction) (Ashkenazi & Dixit, 1998, *Science* 281:1305-1308). These methods are meant to be illustrative only; many other suitable methods are known in the art.

10 In certain embodiments, the step of measuring the amount of FRET is performed by ratiometric analysis. In certain embodiments, the step of measuring the amount of FRET is performed by measuring an increase or decrease in the emission of the donor GFP. In certain embodiments, the step of measuring the amount of FRET is performed by measuring an increase or decrease in the emission of the acceptor GFP.

15 The conditions under which the step of exposing the cells to the substance is practiced are conditions that are typically used in the art for the study of protein-ligand interactions or exposure of cells to compounds: *e.g.*, physiological pH; salt conditions such as those represented by such commonly used buffers as PBS or in tissue culture media; a temperature of about 4°C to about 55°C. In certain 20 embodiments, it may be advantageous to include a substance that quenches background fluorescence in the tissue culture media in which the cells are grown.

25 In certain embodiments, the above-described methods are practiced as methods of high-throughput screening. In particular embodiments, the methods are repeated at least 5,000, preferably at least 50,000, even more preferably at least 100,000 times in  $\pm$  24 hour period. In particular embodiments, at least 5,000, preferably at least 50,000, even more preferably at least 100,000 different substances are tested in a 24 hour period. In particular embodiments, the methods are carried out in microtiter-like plates. In particular embodiments, the microtiter-like plates have 96 wells, 384 wells, 1,536 wells, 3,064 wells, 3,456 wells, or 9,600 wells.

30 In particular embodiments of the methods of identifying a caspase activator or a caspase inhibitor, step (c) does not comprise transfecting the cells with a gene that induces apoptosis or caspase activation and the step of measuring FRET does not include FACS analysis or flow cytometry.

In particular embodiments, the caspase is a mammalian caspase. In particular embodiments, the caspase is a human caspase. In particular embodiments, the caspase is selected from the group consisting of:

caspase-1 (also known as interleukin-1 $\beta$  converting enzyme (ICE))  
5 (Thornberry et al., 1992, Nature 356:768-774; Cerretti et al., 1992, Science 256:97-100);  
caspase-2 (also known as ICH-1) (Wang et al., 1994, Cell 78:739-750);  
caspase-3 (also known as apopain, CPP32, Yama) (Nicholson et al.,  
10 1995, Nature 376:37-43; Rotonda et al., 1996, Nat. Struct. Biol. 3:619-625);  
caspase-4 (also known as ICErel-II, TX, ICH-2) (Munday et al., 1995,  
J. Biol. Chem. 270:15870-15876; Kamens et al., 1995, J. Biol. Chem. 270:15250-15256; Facheu et al., 1995 EMBO J. 14:1914-1922);  
caspase-5 (also known as ICErel-III, TY) ((Munday et al., 1995, J.  
15 Biol. Chem. 270:15870-15876; Faucheu et al., 1996, Eur. J. Biochem. 236:207-213);  
caspase-6 (also known as Mch2) (Fernandes-Alnemri et al., 1995,  
Cancer Res. 55:2737-2742);  
caspase-7 (also known as Mch3, ICE-LAP3, CMH-1) (Fernandes-  
Alnemri et al., 1995. Cancer Res. 55:6045-6052);  
20 caspase-8 (also known as MACH, FLICE, Mch5) (Boldin et al., 1996,  
Cell 85:803-815; Muzio et al., 1996, Cell 85:817-827);  
caspase-9 (also known as ICE-LAP6, Mch6) (Duan et al., 1996, J.  
Biol. Chem. 271:16720-16724);  
caspase-10 (also known as Mch4);  
25 caspase-11; and  
caspase-12.

In particular embodiments, the caspase cleavage site comprises an amino acid sequence selected from the group consisting of:

DXXD (SEQ.ID.NO.:7), where X is any naturally occurring amino  
30 acid;  
DEXD (SEQ.ID.NO.:8), where X is any naturally occurring amino acid;  
DEVD (SEQ.ID.NO.:9);  
DEHD (SEQ.ID.NO.:10);

DETD (SEQ.ID.NO.:11);  
DGPD (SEQ.ID.NO.:12);  
DEPD (SEQ.ID.NO.:13);  
DELD (SEQ.ID.NO.:14);  
5 DAVD (SEQ.ID.NO.:15);  
DMQD (SEQ.ID.NO.:16);  
DSID (SEQ.ID.NO.:17);  
DVPD (SEQ.ID.NO.:18);  
DQTD (SEQ.ID.NO.:19);  
10 DSLD (SEQ.ID.NO.:20);  
WEHD (SEQ.ID.NO.:21);  
ZEXD (SEQ.ID.NO.:22), where X is any naturally occurring amino acid and Z is either I, L, or V;  
LEHD (SEQ.ID.NO.:23);  
15 VEHD (SEQ.ID.NO.:24);  
LETD (SEQ.ID.NO.:25);  
YVAD (SEQ.ID.NO.:26);  
LVAD (SEQ.ID.NO.:27);  
ELPD (SEQ.ID.NO.:28);  
20 SRVD (SEQ.ID.NO.:29); and  
VEID (SEQ.ID.NO.:30).  
IETD (SEQ.ID.NO.:31).

Thromberry et al., 1997, J. Biol. Chem. 272:17907-17911 discloses the cleavage site preferences of a variety of caspases. This publication describes methods 25 of identifying caspase cleavage sites by the use of a positional scanning combinatorial library. In general, suitable caspase cleavage sites for use in the present invention include any sequences that are determined to be caspase cleavage sites by such methods or that are known in the art to be caspase cleavage sites.

In certain embodiments of the above-described methods, the caspase 30 for which an activator or an inhibitor is identified is a caspase that is naturally expressed in the cell. In other embodiments, the caspase for which an activator or an inhibitor is identified is provided by transfecting into the cells an expression vector that directs the expression of the caspase. This can be done in order to provide for expression of the caspase in cells in which the caspase is not naturally expressed (e.g.,

in yeast or *C. elegans*, which have no endogenous caspases) or this can be done in order to provide a higher level of expression of the caspase in cells in which the caspase is naturally expressed at low levels. In a variation of this approach, the caspase that is transfected into the cells is a caspase that contains mutations or 5 alterations in its amino acid sequence as compared to a wild-type caspase. In another variation, the caspase is derived from a species other than the species from which the cells are derived.

10 While the above-described methods are explicitly directed to testing whether "a" substance is an activator or inhibitor of a caspase, it will be clear to one skilled in the art that such methods can be adapted to test collections of substances, *e.g.*, combinatorial libraries, collections of natural products, *etc.*, to determine whether any members of such collections are activators or inhibitors of the caspase. Accordingly, the use of collections of substances, or individual members of such 15 collections, as the substance in the above-described methods is within the scope of the present invention.

Once a substance has been identified as an activator or an inhibitor of a caspase by the above-described methods, it may be desirable to determine whether 20 that substance acts at the level of caspase proteolysis rather than at the level of upstream events that activate the caspase. This can be done, *e.g.*, by setting up *in vitro* assays in which purified caspase is incubated with purified fusion protein. In such *in vitro* assays, the absence of other components of the apoptotic pathway means that any effect caused by the substance on caspase activity (as measured by changes in FRET) will be due to a direct effect on the caspase itself. An example of such an *in vitro* assay is described in Examples 2 and 9 and Figure 4 herein.

25 Another way to determine the level at which a caspase inhibitor acts is to allow a period of time to pass between step (b) of the method (treating the cells in order to activate a caspase) and step (d) (exposing the cells to a substance suspected of being a caspase inhibitor). This period of time will allow for active caspase to be generated. This active caspase will result in a large amount of cleavage of the fusion 30 protein, and thus little FRET. If the substance is an inhibitor, then exposing the cells to the substance will increase the amount of FRET. If the inhibitor acts at the level of caspase proteolysis, this increase should occur quickly, on the order of the amount of time it takes the inhibitor to enter the cells. If the inhibitor acts on upstream events, *e.g.*, if it acts to turn off upstream caspase activation events, then the effect of the

inhibitor on caspase proteolysis should occur more slowly. In such a case, the increase in FRET should occur more slowly as well.

The amount of time it takes for the treatment of step (b) to actually activate the caspase will vary depending upon a number of factors such as the type of cell used in the method, the nature of the treatment (e.g., the identity of a compound used to activate the caspase), and the concentration of the compound that is used to activate the caspase. Some compounds can activate a caspase and/or induce apoptosis very quickly (*i.e.*, on the order of minutes), while others take hours or even overnight incubation. In most cases, the treatment that activates the caspase should be carried out for about 2 to 8 hours before the cells are exposed to the substance suspected of being a caspase inhibitor, if it is desired to obtain knowledge of the level at which the substance acts. However, in some embodiments, the cells may be exposed to the substance concurrently with or prior to the application of the treatment that activates a caspase. This will depend on factors such as the metabolic stability of the substance, cell permeability, or even the kinetic characteristics of the substance/caspase interaction, *e.g.*, the on rate, affinity,  $K_i$ . One skilled in the art will be able to readily determine the proper relationship between the treatment and exposure to the substance, depending upon the particular details of the method as it is practiced.

Measuring the amount of FRET is generally done by exciting the donor GFP by use of a lamp, laser, or other light source tuned to the donor GFP's excitation wavelength. The excitation energy absorbed by the donor can be emitted by the donor at its emission wavelength or the excitation energy can be transferred, at least partially, to the acceptor GFP which can in turn emit the excitation energy at its own emission wavelength. By measuring the amount of emission at both the acceptor and donor wavelengths, one can determine how much FRET has occurred. A high ratio of acceptor/donor emission signifies a high amount of FRET; conversely, a low ratio of acceptor/donor emission signifies a low amount of FRET.

This use of ratiometric analysis is preferred because the magnitude of the ratio of donor acceptor emission is a measure of the cleavage of the fusion protein that is not influenced by such factors as the absolute amount of the fusion protein or its cleaved products, the optical thickness of the sample being assayed, the brightness of the excitation source, or the sensitivity of the detector.

The use of ratiometric analysis also is preferred because ratiometric analysis affords a level of sensitivity that is suited for use in high-throughput screening methods.

The sensitivity of the methods of the present invention is also increased by the use of ultra-bright GFPs as donor GFPs. Rather than employing ultra-bright GFPs, the prior art employed a type of GFP known as a blue fluorescent protein (BFP) as a donor GFP (Xu et al., 1998, *Nucl. Acids Res.* 26:2034-2035 (Xu)). BFPs have a mutation at position 66 that changes the wild-type tyrosine at that position to a histidine. This Y66H change results in a protein having relatively weak fluorescence (*i.e.*, a fluorescence quantum yield of about 0.30 or less). While such weak fluorescence may be suitable for flow cytometry (*e.g.*, FACS analysis, as in Xu), it is much less suitable for ratiometric analysis, and therefore is also much less suitable for use in high-throughput screening. The fusion protein described in Xu is very unlikely to provide a sensitive enough readout to be useful in the high-throughput screening methods of identifying caspase inhibitors carried out in microtiter-like plates that are a preferred embodiment of the present invention. Careful analysis of Xu reveals that Xu did not disclose methods of identifying caspase inhibitors that utilize FRET. The only method in which Xu employed a caspase inhibitor involved the use of Western blotting to identify fragments of Xu's fusion protein. In the presence of a caspase inhibitor, Xu was able to demonstrate that production of the fragments was abolished (see Figure 1B, page 2035). Where Xu used fluorescence measurements (Figure 2, page 2035), Xu was limited to measuring the effects of caspase activation rather than inhibition.

The difficulty of using BFPs for ratiometric analysis is especially pronounced in living cells. See Pollock & Heim, 1999, *Trends Cell Biol.* 9:57-60, at page 58, for a discussion of this issue. In view of this difficulty, preferred embodiments of the present invention make use of a donor GFP that does not have a histidine at position 66, *i.e.*, a GFP that does not have the Y66H change.

High-throughput screening, as understood herein, requires sample throughputs that exceed the present capacity of such methods as flow cytometry. Flow cytometry requires such steps as harvesting cells, washing the cells, feeding the cells into a suitable apparatus, and then analyzing the cells one at a time. High-throughput screening is preferably done in multi-well microtiter-like plates rather than in flow cytometry instrumentation. Multi-well plates afford the possibility of parallel

analysis of thousands of assays every few minutes, whereas flow cytometry permits the analysis of at most hundreds of assays per minute (see Nolan et al., 1999, Drug Discovery Today 4:173-180, at page 179, right column). A variety of automated analysis equipment that can be used with microtiter-like plates and that is suitable for carrying out high-throughput screening is known. See, e.g., U.S. Patent No. 5,670,113; U.S. Patent No. 5,139,744; and U.S. Patent No. 4,626,684.

The methods of the present invention provide several advantages compared to the prior art that make these methods especially suitable for high-throughput screening. The use of ratiometric analysis with ultra-bright GFPs obviates the cumbersome and time consuming step of harvesting cells from the multi-well plates, as required in flow cytometry methods such as, e.g., FACS analysis. Thus, the methods of the present invention are especially suitable for use in adherent cells such as Hela cells, 293 cells, L cells, CHO cells, PC12 cells, COS cells, or 3T3 cells. The methods described herein, by obviating the need for such cumbersome steps as harvesting adherent cells from the multi-well plates, provide means of screening vastly more compounds than can be done using less efficient flow cytometry methods, such as those described in Xu et al., 1998, Nucl. Acids Res. 26:2034-2035. Even when the methods of the present invention are used with suspension cells, the methods have the advantage over prior art methods that the suspension cells need not be harvested before they are assayed for FRET.

The prior art used a linker region of 18 amino acids in a fusion protein comprising blue and green fluorescent proteins with a caspase cleavage site (Xu et al., 1998, Nucl. Acids Res. 26:2034-2035 (Xu)). The use of such a long linker region was believed by Xu to be necessary to "make this region soluble and accessible to cleavage by CPP32." (page 2034, right column, top). The present inventors have discovered that, contrary to the teachings of the prior art, a shorter linker region (e.g., 10, 9, 8, 7, 6, 5, or 4 amino acids) is more effective than the longer linkers disclosed in the prior art. See Figure 1B, where the linker region for the fusion protein WT-1x is shown as being only 9 amino acids long. The present inventors have demonstrated, contrary to what might be expected based upon the prior art, that a linker as short as this is sufficient to permit cleavage by caspases as well as efficient FRET measurement. See Figure 4B, where the data for WT-1x show that caspase cleavage of WT-1x can be easily detected in living cells using ratiometric analysis. A comparison of Figure 4B and Figure 4A points out the value of the present inventors'

discovery of the use of such ratiometric analysis in the context of short linkers. Although Western blot analysis of Hela cells expressing WT-1x that have been induced to undergo apoptosis shows only a tiny amount of WT-1x cleavage product, which could be easily missed, ratiometric analysis of the same cells clearly shows that 5 cleavage has occurred (the FRET ratio drops by more than 50%). Thus, the use of ratiometric analysis affords much greater sensitivity to assays for caspase activity than does the use of Western blotting. Of course, such increased sensitivity is of great value in the use of such assays to identify activators of the expression of caspases and inhibitors of caspase activity.

10 The present invention includes a fusion protein and its use in the methods described herein where all of the amino acids in the fusion protein are derived from the amino acids of the donor and acceptor GFPs except for a four or five amino acid linker that is a caspase cleavage site. In some embodiments, it may be advantageous to use a glycine or other amino acid with a small side chain on the 15 prime side (*i.e.*, in the position immediately after the aspartate cleavage position) in order to avoid steric hinderance of the caspase.

The inhibitors of caspases identified by the methods of the present invention will have a variety of uses. Such inhibitors will be useful in treating those conditions where it is beneficial to decrease the level of caspase activity or apoptosis. 20 Such conditions include, *e.g.*, neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease; traumatic brain injury; stroke; ischemia-reperfusion injury; graft-versus-host disease; and autoimmune disorders. It is clear that those skilled in the pharmaceutical arts view caspase inhibitors as having useful pharmacological activities. An inhibitor of caspase-1 (interleukin-1 $\beta$  converting enzyme) is currently 25 in clinical trials for the treatment of inflammatory diseases (Press release, Vertex Pharmaceuticals, Inc., October 29, 1998). A caspase inhibitor has been shown to reduce neuronal damage in a rabbit model of bacterial meningitis (Braun et al., 1999, Nature Medicine 5:298-302).

The activators of caspases identified by the methods of the present 30 invention will likely be useful in the treatment of conditions where it is beneficial to increase the level of caspase activity or apoptosis, *e.g.*, cancer or any disease that involves hyperplasia or neoplastic transformation, graft versus host disease. A caspase activator/inducer of apoptosis is in clinical trials for use against graft versus host disease (Press release, Ariad Pharmaceuticals, May 6, 1999).

The present invention includes pharmaceutical compositions comprising activators and inhibitors of caspases that have been identified by the above-described methods. The activators and inhibitors are generally combined with pharmaceutically acceptable carriers to form pharmaceutical compositions. Examples 5 of such carriers and methods of formulation of pharmaceutical compositions containing activators and inhibitors and carriers can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain a therapeutically effective amount of the activators and inhibitors.

10 Therapeutic or prophylactic compositions are administered to an individual in amounts sufficient to treat or prevent conditions where caspase activity is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, gender, and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician.

15 Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

20 The compositions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

25 Advantageously, compositions can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To 30 be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age, weight; sex and

medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular composition thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of composition within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

10

The following non-limiting examples are presented to better illustrate the invention.

#### EXAMPLE 1

15 Fusion proteins comprising a Group II caspase cleavage site

Recombinant caspase substrates were engineered to be encoded as inducible fusion proteins comprised of tandem green fluorescent protein (GFP) molecules tethered by amino acid sequences that are recognized and cleaved by group II caspases (e.g., caspase-3) (Figure 1B) (Thornberry et al., 1997, J. Biol. Chem. 272:17907-17911). A tetracycline-inducible expression system was used to circumvent potential problems with non-specific GFP-mediated toxicity due to prolonged overexpression (Gossen et al., 1994, Curr. Opin. Biotechnol. 5:516-520).

20 The caspase recognition motif (DEVD (SEQ.ID.NO.:9)) embedded within these fusion proteins was derived from the protease cleavage site identified in poly-(ADP-ribose)- polymerase (PARP), a well characterized substrate of caspase-3 in apoptotic cells (Lazebnik et al., 1994, Nature 371:346-347). Furthermore, this site has been defined as the optimal group II caspase cleavage motif on the basis of studies that have examined cleavage of combinatorial fluorogenic tetrapeptide substrate libraries (Thornberry et al., 1997, J. Biol. Chem. 272:17907-17911).

25 30 The spectral properties (excitation and emission) of the mutant GFP proteins (W1B, Topaz; Aurora Biosciences, San Diego, CA) used to generate the fusions are well suited for discrimination between the cleaved and uncleaved forms of

the fusion protein using standard fluorometry (Tsien, 1998, *Annu. Rev. Biochem.* 67:509-544). W1B has dual excitation (434/452 nm) and emission peaks (476/505 nm), while Topaz has and excitation and emission maxima of 514 nm and 527 nm, respectively. In principle, caspase-3-mediated cleavage of these substrates should 5 prevent fluorescence resonance energy transfer (FRET) between linked GFP proteins due to physical separation of the molecules. When excitation occurs at the excitatory wavelength (ex: 434 nm) of the donor molecule, cleavage of the substrate can be quantitated as the ratio of excited fluorescence emitted by the acceptor molecule (Topaz, em: 527 nm) divided by the fluorescence emitted by the donor molecule (W1B, em: 476 nm). In the cell-based assays described herein, an increase in the ratio 10 of emitted fluorescence at these wavelengths (527/476) would reflect an inhibition of caspase activity.

In order to successfully develop this assay, various criteria had to be realized. For example, it was necessary to demonstrate that the caspase-3 cleavage 15 motif embedded within the linker region could be efficiently cleaved by caspase-3 without impairing FRET between the tethered molecules. To this end, several potential linker regions comprising single or tandem DEVD (SEQ.ID.NO.:9) sites were designed in order to optimize both cleavage as well as FRET efficiency. A construct, designated WT-2xL was generated in which tandem DEVD 20 (SEQ.ID.NO.:9) sites were flanked by hinge regions, rich in glycine and serine residues, that could impart flexibility to the GFP fusion protein and promote energy transfer (Hall, 1995, *J. Parenter Enteral. Nutr.* 22:393-398; Freund et al., 1993, *FEBS Lett.* 320: 97-100; Steinert et al., 1991, *Int. J. Biol. Macromol.* 13:130-139). Figure 2A-B shows a direct correlation between the length of the linker region examined and 25 the efficiency of FRET in transfected Hela cells overexpressing the fusion proteins. As the length of the linker separating the GFP molecules was increased, a corresponding decrease in FRET efficiency was observed. Energy transfer was more efficient in fusions containing the single (WT-1x) versus the tandem (WT-2x) DEVD (SEQ.ID.NO.:9) motif. Interestingly, inclusion of a flexible hinge region within the 30 linker offered no particular advantage, but instead served to diminish FRET efficiency of the WT-2xL fusion protein relative to its parental form (WT-2x).

## EXAMPLE 2

*In vitro expression and cleavage of fusion proteins comprising a Group II caspase cleavage site*

The sensitivity of the tandem GFP substrates to cleavage by caspases was examined initially using [<sup>35</sup>S]-Met radiolabeled proteins translated in wheat germ lysates. The proteins were incubated *in vitro* with increasing concentrations of purified recombinant caspase-3 and the cleavage products were resolved by polyacrylamide gel electrophoresis and quantitated by autoradiographic analysis of the gels. A comparison of the single (WT-1x) versus the dual DEVD (SEQ.ID.NO.:9)-containing substrate (WT-2x) showed the latter to be cleaved approximately four-fold more efficiently as estimated by their respective  $K_{cat}/K_m$  values (Figure 3). This result is consistent with data using other recombinant caspase substrates in which artificial DEVD (SEQ.ID.NO.:9) motifs have been artificially introduced (unpublished observations). This also demonstrates that, by multimerizing DEVD (SEQ.ID.NO.:9) motifs, it is possible to generate artificial substrates that are cleaved as efficiently as authentic group II caspase substrates (*e.g.* PARP, U1-70 kDa, and DNA-PKcs) (Casciola-Rosen et al., 1996, *J. Exp. Med.* 183:1957-1964). To compare the efficiencies with which these fusion proteins could be cleaved by caspases in intact cells, Hela cells transfected with the various constructs were stimulated to undergo apoptosis and the cleavage products were analyzed by immunoblot analysis using polyclonal anti-GFP antibodies (Figure 4A). Several studies have previously demonstrated that staurosporine induces proteolytic activation of caspase-3 in Hela cells. In contrast to the negative influence on FRET efficiency conferred by increasing the number of DEVD (SEQ.ID.NO.:9) sites in the linker region separating the two GFP molecules, fusion proteins harboring tandem DEVD (SEQ.ID.NO.:9) motifs were cleaved more efficiently following staurosporine treatment. Furthermore, cells transfected with the parental construct lacking a caspase-3 cleavage site (WT) failed to induce proteolytic cleavage of the fusion protein in response to apoptosis. In addition, cleavage of the DEVD (SEQ.ID.NO.:9)-containing fusion proteins was dramatically attenuated by incubating staurosporine-treated cultures with zVAD-fmk, an irreversible pan-caspase peptide inhibitor. This data confirmed that recombinant

GFP substrates could be used to monitor activation as well as inhibition of group II caspase in cells.

### EXAMPLE 3

5 Expression and cleavage of fusion proteins comprising a Group II caspase cleavage site in living cells

To determine whether inhibition of caspase cleavage could cause a measurable and dose-dependent change in FRET, caspase inhibition by zVAD-fmk was titrated in cultured HeLa cells stably overexpressing the WT-1x construct. This 10 fusion construct was chosen for analysis because it represented a reasonable compromise between energy transfer efficiency and cleavability as a substrate (Figure 2A-B and Figure 3). Caspase inhibition was examined using intact adherent cells plated in a 96 well microtiter-like plate format. The fluorometer, equipped with the appropriate filter sets to detect FRET, was calibrated to read fluorescence emitted 15 from intact cells lying on the surface of the plate. Following treatment with staurosporine alone or in combination with different concentrations of the inhibitor (zVAD-fmk), the cells were examined by fluorescence analysis. The data in Figure 5 show that treatment of WT-1x-expressing cells with increasing concentrations of zVAD-fmk resulted in a concomitant and dose-dependent increase in WT-1x-derived 20 FRET ( $IC_{50} = 23 \mu M$ ). The dose-response curve is similar to that obtained using other biochemical markers of apoptosis, including DNA fragmentation (not shown). These results demonstrate that quantitation can be performed directly on cells without 25 a need for further manipulation (e.g. cell harvesting, lysate preparation). Furthermore, the ability to perform the assay in a multi-well format emphasizes its usefulness as a screening tool for drug discovery.

### EXAMPLE 4

High-throughput screening

To explore further the utility of the tandem GFP assay as a high 30 throughput screening methodology for identifying caspase inhibitors, duplicate sets of

96 well drug plates containing either single compounds or mixtures of ten compounds per well were tested. The final concentration of each drug in these plates ranged between 400-600  $\mu$ M. Pre-screening of these plates revealed the absence of caspase inhibitors among the collection of test compounds (data not shown). In order to

5 demonstrate that caspase inhibitors would have been detected, had they been present among these plates, individual wells were supplemented with different concentrations of a known caspase inhibitor (zVAD-fmk). Furthermore, to avoid any bias in the interpretation of the data, the experiment was performed blindly, such that the location of individual wells containing zVAD-fmk was not revealed until the

10 experiment was completed. In these experiments, two wells in each plate were randomly supplemented with three different concentrations of zVAD-fmk (2.5 $\mu$ M, 25 $\mu$ M, and 250 $\mu$ M) and each plate was assayed in duplicate. The screening results are shown in Figure 6A-B, where Figure 6A and Figure 6B represent plate arrays containing the single compounds and drug mixtures, respectively. An examination of

15 the data showed that, regardless of the plate format, duplicate wells containing either 25  $\mu$ M or 250  $\mu$ M zVAD-fmk were readily identified. As expected, zVAD-fmk could not be detected at a concentration (2.5 $\mu$ M) which fails to inhibit cleavage of the tandem GFP substrate (see Figure 5). In one of the plates containing individual compounds, a single well (coordinates G3) produced a positive signal that was not

20 attributable to the presence of zVAD-fmk. This sample, however, represented a false-positive result as it did not register as a hit on a duplicate plate. Only one additional false-positive hit, at the same well position (coordinates G3), was detected on the plate containing the drug mixtures. Although this sample scored as a positive hit on duplicate plates, the fluorescence emission values recorded at both the 490 nm or 530

25 nm wavelengths, were far in excess of the maximum emission values obtained from the control wells, indicating that this false-positive hit represented a highly fluorescent compound(s). Finally, while the false-positive hit rate was very low (0.1% - 1.0%), it is important to note that both false-positive hits detected in our assay could have easily been filtered out through the use of data analysis algorithms

30 designed to flag such hits.

## EXAMPLE 5

Constructs

The W1B GFP mutant was amplified by polymerase chain reaction (PCR) as an EcoRI/W1B/AflII/AgeI fragment from pRSETA-hW1B (Aurora Biosciences, San Diego, CA). A Kozak consensus (gccgccaccatgg; SEQ.ID.NO.:32) was engineered into the start methionine of W1B and the stop codon was deleted (Kozak, 1984, Nucleic Acids Res. 12:857-872). Topaz was amplified by PCR as an AgeI/NheI/TOPAZ/BamHI fragment from pRSETA-TOPAZ (Aurora Biosciences, San Diego, CA). The start methionine of Topaz was deleted. W1B and Topaz were co-ligated into the EcoRI/BamHI sites of pIRES1neo (Clontech, Palo Alto, CA) as EcoRI/AgeI and AgeI/BamHI fragments respectively to generate pIRES1neo-WT. This created a tandem fusion of W1B and TOPAZ separated by a six amino acid linker encoded by the AflII/AgeI/NheI sites. pIRES1neo-WT-1x, pIRES1neo-WT-2x, and pIRES1neo-WT-2xL were generated by ligation of synthetic oligonucleotides encoding DEVD (SEQ.ID.NO.:9) linker sequences into the AflII/NheI sites of the parental pIRES1neo-WT plasmid.

## EXAMPLE 6

Transfections

Heia cells ( $7.5 \times 10^5$ ) were subcultured into 60 mm tissue culture dishes and transfected the following day with 2.5  $\mu$ g of plasmid DNA using the Lipofectamine Plus reagent (Life Technologies). Cells were allowed to express the GFP fusions for a further 48 hours prior to analysis. To determine maximum possible FRET efficiency, zVAD-fmk (100  $\mu$ M) (Enzyme Systems Products) was included in the media during the transfection procedure and maintained for the 48 hours in order to inhibit caspase activity that is generated as a consequence of the transfection. In the Western blotting experiments, zVAD-fmk was included in the media as described above. For induction of apoptosis, cells were treated with staurosporine (1  $\mu$ M) for 4 hours following the initial 48 hour expression period.

30

## EXAMPLE 7

Preparation of lysates

HeLa cells from 60 mm tissue culture dishes were scraped into their growth media and pelleted at 2,000 x g for 10 minutes at 4°C. Cell pellets were 5 rinsed with 1 ml cold PBS and repelleted as described above. One hundred microliters of TET buffer (50 mM Tris, pH 7.5/2 mM EDTA/1 % Triton X-100) was used to disrupt the cell pellets and the resulting lysates were cleared by centrifugation at 10,000 x g for 10 minutes at 4°C. For IC<sub>50</sub> determinations, 1 μM DEVD (SEQ.ID.NO.:9)-CHO (Biomol) was included in the TET buffer in order to abolish 10 DEVD (SEQ.ID.NO.:9)-caspase activity following cell lysis.

## EXAMPLE 8

FRET measurements

Lysates corresponding to 1 mg of total protein (transient transfections) 15 or complete lysate (stable cell lines) were diluted to 1 ml with 50 mM Tris, pH 7.5 and the fluorescence was measured using a Perkin Elmer LS 50B fluorometer. The ratio of the intensity of the 527 nm emission peak (TOPAZ) over the intensity of the 476 nm emission peak (W1B) after excitation at 434 nm (W1B) provided us with a measurement of the level of FRET occurring between the two GFP chromophores. 20 No fluorescence was detected in HeLa cells transfected with the empty pIRES1neo vector.

## EXAMPLE 9

In vitro cleavage of tandem GFP substrates

25 The WT-1x and WT-2x coding sequences were subcloned from pIRES1neo into pCDNA3.1(-) (Invitrogen, Palo Alto, CA) using existing EcoRI/BamHI sites. [<sup>35</sup>S]methionine-labeled WT-1x and WT-2x proteins were generated using a coupled T7-wheat germ *in vitro* transcription/translation system

(Promega). pcDNA3.1(-) constructs (2  $\mu$ g) were incubated with 8  $\mu$ l of [ $^{35}$ S]methionine (10  $\mu$ Ci/ $\mu$ l) (Amersham) and 2  $\mu$ l RNAsin (40 U/ $\mu$ l) (Boehringer Mannheim) in a total of 100  $\mu$ l of wheat germ lysate for 45 minutes at 30°C. Cleavage of radiolabeled WT-1x or WT-2x protein was performed by incubation with 5 purified recombinant caspase-3 in a final volume of 10  $\mu$ l of cleavage buffer (50 mM HEPES/KOH (pH 7.0), 2 mM EDTA, 0.1% CHAPS, 10% (w/v) glycerol, 10 mM DTT) for 1 hour at 3°C. The reactions were terminated by addition of SDS sample buffer followed by heating to 95°C for 5 minutes. The cleavage products were resolved by SDS-poly acrylamide gel electrophoresis and visualized by 10 autoradiography. Autoradiograms were quantitated by laser scanning densitometry.

#### EXAMPLE 10

##### Western blots

Crude cell lysates (15 mg) were resolved on 10-20% Tris-glycine SDS 15 gels and transferred to nitrocellulose membranes as previously described (Rasper et al., 1998, Cell Death and Differentiation 5:271-288). Immunoblot analysis was performed using a 1:500 dilution of a rabbit polyclonal anti-GFP antibody (Clontech, Palo Alto, CA). A 1:3000 dilution of donkey anti-rabbit peroxidase (Amersham) was used as the secondary antibody. Protein bands were visualized by ECL detection 20 (Amersham) and autoradiography.

#### EXAMPLE 11

##### Generation of stable cell lines and 96-well plate assays

To generate stable cell clones capable of high level expression of the 25 tandem GFP substrate, a tetracycline-inducible expression system was used (Clontech, Palo Alto, CA). Following cotransfection of HeLa Tet-On cells (Clontech, Palo Alto, CA) with pTRE-WT-1x and pTK-Hyg (Clontech, Palo Alto, CA) in a 60 mm dish, the cells were grown for a further 48 hours before being subcultured into 100 mm dishes in the presence of 150  $\mu$ g/ml hygromycin. Pooled hygromycin- 30 resistant colonies were treated with 2  $\mu$ g/ml doxycycline for 48 hours to induce

expression of the WT-1x fusion protein. Fluorescent clones were individually isolated into a 96-well plate using fluorescence activated cell sorting (FACS). One cell clone, HETON-WT-1x-57, was chosen for further analysis and was plated into a Costar black-walled 96-well clear-bottomed plate at a density of 4,000 cells/well in  
5 the presence of 2  $\mu$ g/ml doxycycline. After two days of incubation, the media was replaced with fresh media containing doxycycline (200  $\mu$ l/well). Staurosporine and varying concentrations of zVAD-fmk were co-administered to the cells (1.5% final DMSO) and allowed to incubate for 4 hours. The media was subsequently exchanged for PBS and the fluorescence from intact cells measured using a 96 well fluorometer  
10 (Perceptive Biosystems Cytofluor) equipped with the appropriate filter sets (420/50ex, 490/40em. and 530/25em).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in  
15 addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

20

## WHAT IS CLAIMED:

1. A fusion protein comprising a donor green fluorescent protein (GFP) that is an ultra-bright GFP and an acceptor GFP linked by a peptide comprising at least one caspase cleavage site.
2. The fusion protein of claim 1 where the peptide comprising at least one caspase cleavage site contains fewer than 10 amino acids and the acceptor/donor fluorescence resonance energy transfer ratio is at least about 1.5.
3. DNA encoding the fusion protein of claim 1.
4. A method of identifying a caspase activator comprising:
  - (a) providing cells comprising a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site;
  - (b) measuring the amount of fluorescence resonance energy transfer (FRET) in the cells in the absence of a substance suspected of being an activator of the expression of a caspase;
  - (c) exposing the cells to the substance suspected of being an activator of the expression of the caspase;
  - (d) measuring the amount of FRET in the cells in the presence of the substance suspected of being an activator of the expression of the caspase; where if the amount of FRET measured in step (b) is greater than the amount of FRET measured in step (d), then the substance is a caspase activator.
5. A method of identifying a caspase inhibitor comprising:
  - (a) providing cells comprising a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site;
  - (b) treating the cells in order to activate a caspase;
  - (c) measuring the amount of fluorescence resonance energy transfer (FRET) in the treated cells in the absence of a substance suspected of being a caspase inhibitor.

- (d) exposing the treated cells to the substance suspected of being a caspase inhibitor;
- (e) measuring the amount of FRET in the treated cells that have been exposed to the substance suspected of being a caspase inhibitor;
  - where if the amount of FRET measured in step (e) is greater than the amount of FRET measured in step (c), then the substance is a caspase inhibitor.

6. A method of high throughput screening to identify a caspase inhibitor comprising:

repeating at least 5,000 times in a 24 hour period the steps of:

- (a) providing cells comprising a fusion protein comprising a donor and an acceptor green fluorescent protein (GFP) linked by a peptide comprising at least one caspase cleavage site;
- (b) treating the cells in order to activate a caspase;
- (c) measuring the amount of fluorescence resonance energy transfer (FRET) in the treated cells in the absence of a substance suspected of being a caspase inhibitor;
- (d) exposing the treated cells to the substance suspected of being a caspase inhibitor;
- (e) measuring the amount of FRET in the treated cells that have been exposed to the substance suspected of being a caspase inhibitor;
  - where if the amount of FRET measured in step (e) is greater than the amount of FRET measured in step (c), then the substance is a caspase inhibitor;
  - where the treated cells are exposed to at least about 5,000 different substances in the 24 hour period.

7. The method of claim 6 where the cells are eukaryotic cells.

8. The method of claim 6 where the cells are mammalian cells.

9. The method of claim 6 where the cells are selected from the group consisting of: L cells L-M(TK<sup>-</sup>) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), HEK293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61),

3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), Hela (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), PC12 cells, *C. elegans* cells, zebrafish cells, *Xenopus* oocytes, *Xenopus* melanophores, and *Saccharomyces cerevisiae* cells.

10. The method of claim 6 where the donor GFP is an ultra-bright GFP, FRET is measured by ratiometric analysis, the cells are adherent mammalian cells, and the peptide comprising a caspase cleavage site contains fewer than 10 amino acids.

11. The method of claim 10 where the caspase cleavage site is selected from the group consisting of:

D<sub>XX</sub>D (SEQ.ID.NO.:7), where X is any naturally occurring amino acid;

DEXD (SEQ.ID.NO.:8), where X is any naturally occurring amino acid;

DEVD (SEQ.ID.NO.:9);

DEHD (SEQ.ID.NO.:10);

DETD (SEQ.ID.NO.:11);

DGPD (SEQ.ID.NO.:12);

DEPD (SEQ.ID.NO.:13);

DELD (SEQ.ID.NO.:14);

DAVD (SEQ.ID.NO.:15);

DMQD (SEQ.ID.NO.:16);

DSID (SEQ.ID.NO.:17);

DVPD (SEQ.ID.NO.:18);

DQTD (SEQ.ID.NO.:19);

DSLD (SEQ.ID.NO.:20);

WEHD (SEQ.ID.NO.:21);

ZEXD (SEQ.ID.NO.:22), where X is any naturally occurring amino acid and Z is either L, I, or V;

LEHD (SEQ.ID.NO.:23);

VEHD (SEQ.ID.NO.:24);

LETD (SEQ.ID.NO.:25);

YVAD (SEQ.ID.NO.:26);  
LVAD (SEQ.ID.NO.:27);  
ELPD (SEQ.ID.NO.:28);  
SRVD (SEQ.ID.NO.:29);  
VEID (SEQ.ID.NO.:30); and  
IETD (SEQ.ID.NO.:31).

12. The method of claim 10 where the caspase cleavage site is a caspase-2, caspase-3, or caspase-7 cleavage site.

13. The method of claim 10 where:

the ultra-bright GFP is selected from the group consisting of: W1B; TOPAZ; 10C; 10C Q69K; W7; W1C; wild-type GFP having the changes S65G, S72A, and T203F; and wild-type GFP (SEQ.ID.NO.:6) having the changes S65G, S72A, and T203H;

the method is carried out in microtiter-like plates having 96, 384, or 1,536 wells per plate and the volumes of the wells are less than 25  $\mu$ l, less than 25  $\mu$ l, or less than 10  $\mu$ l.

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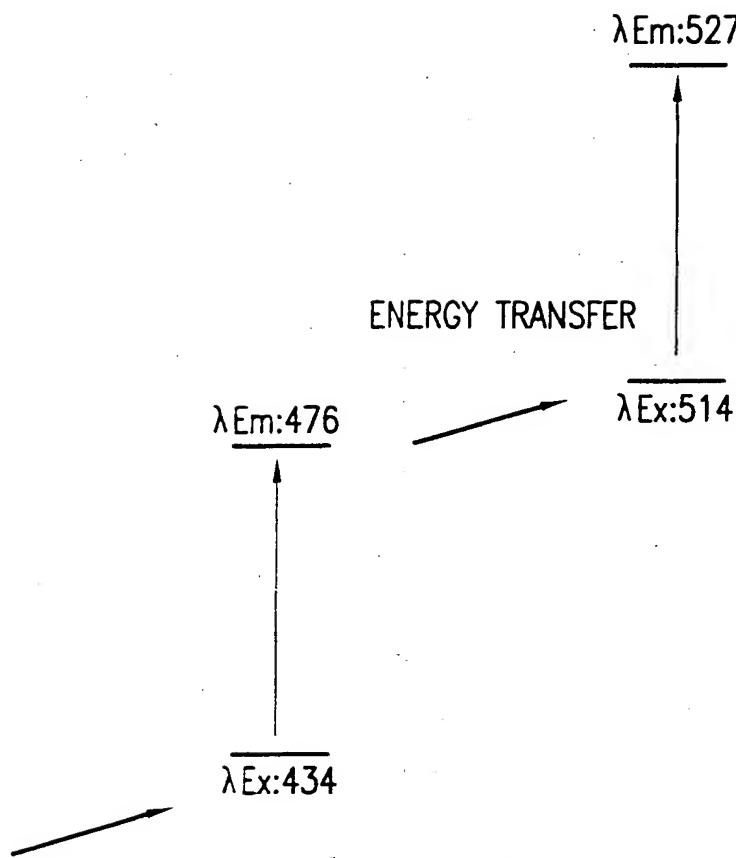


FIG. 1A

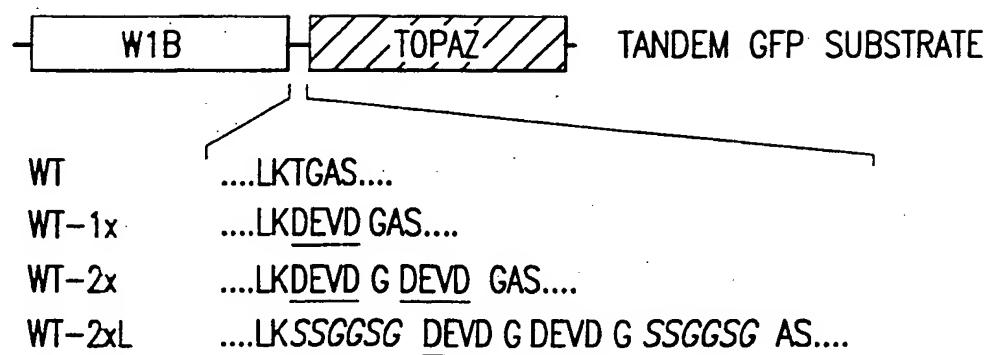


FIG. 1B

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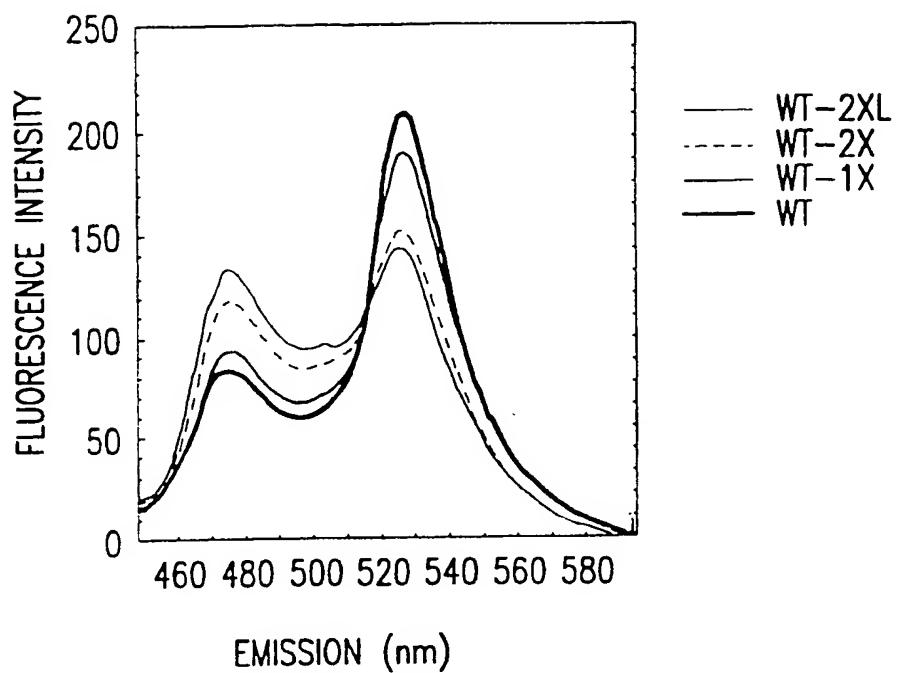


FIG. 2A

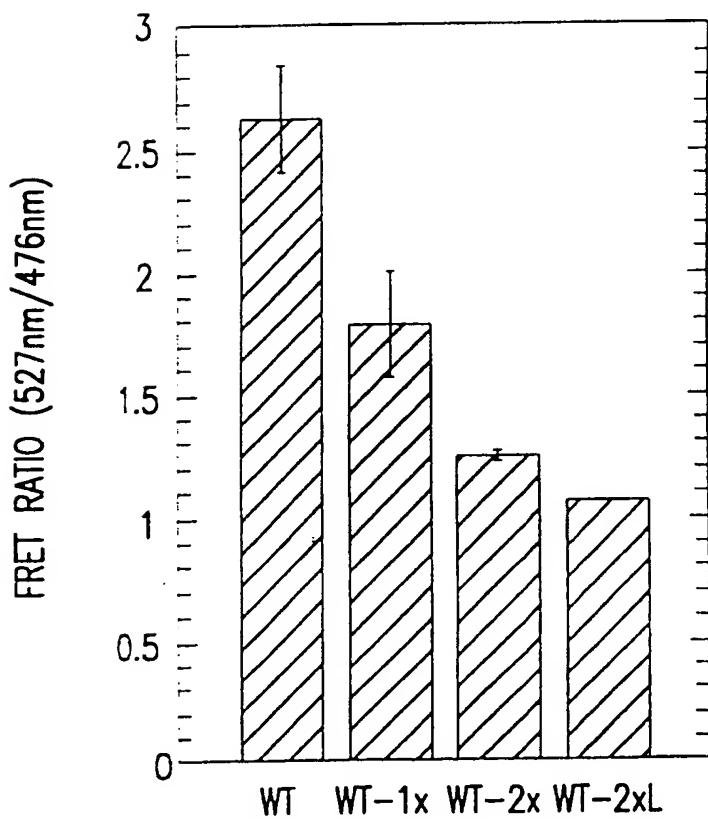


FIG. 2B

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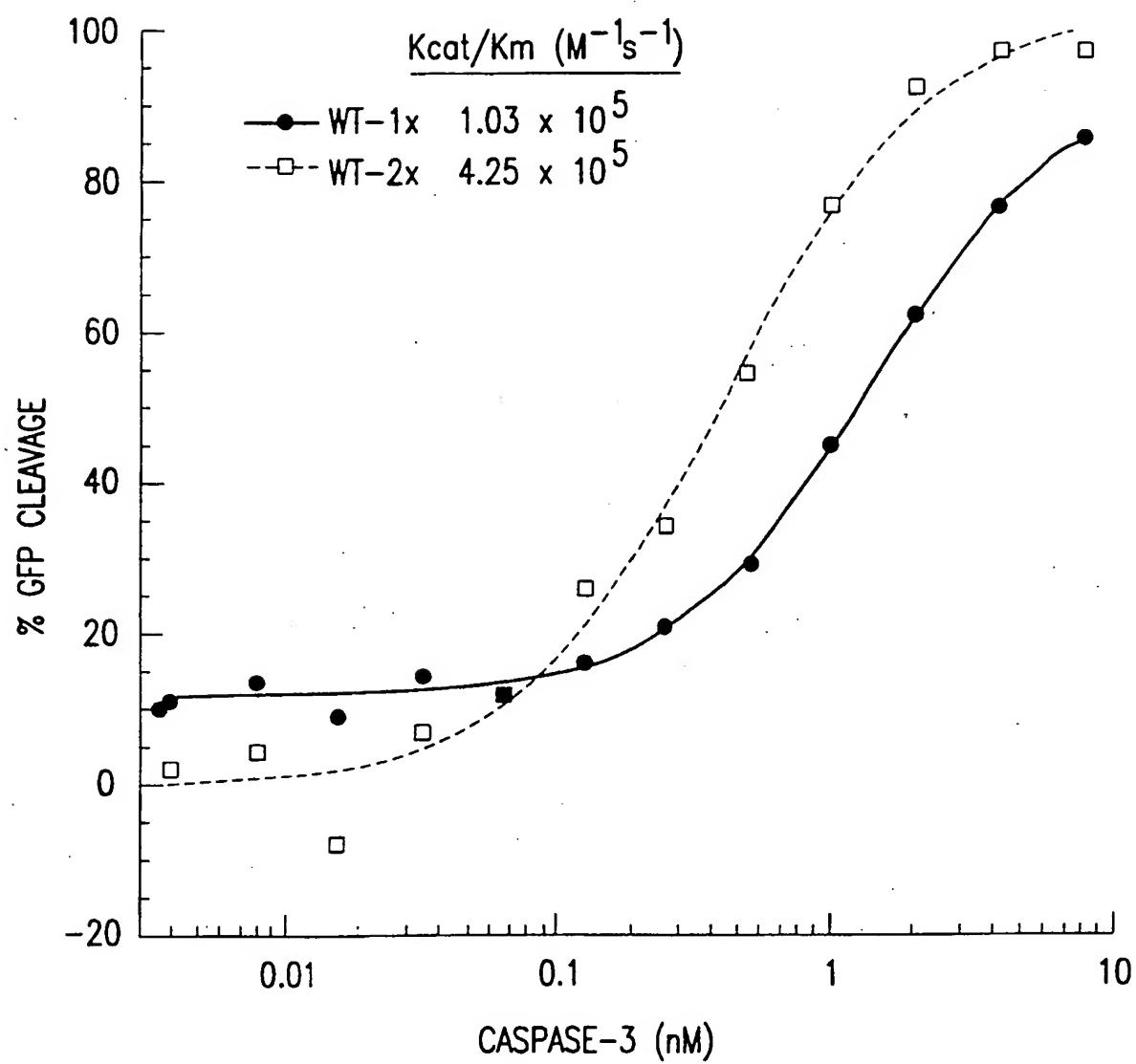


FIG.3

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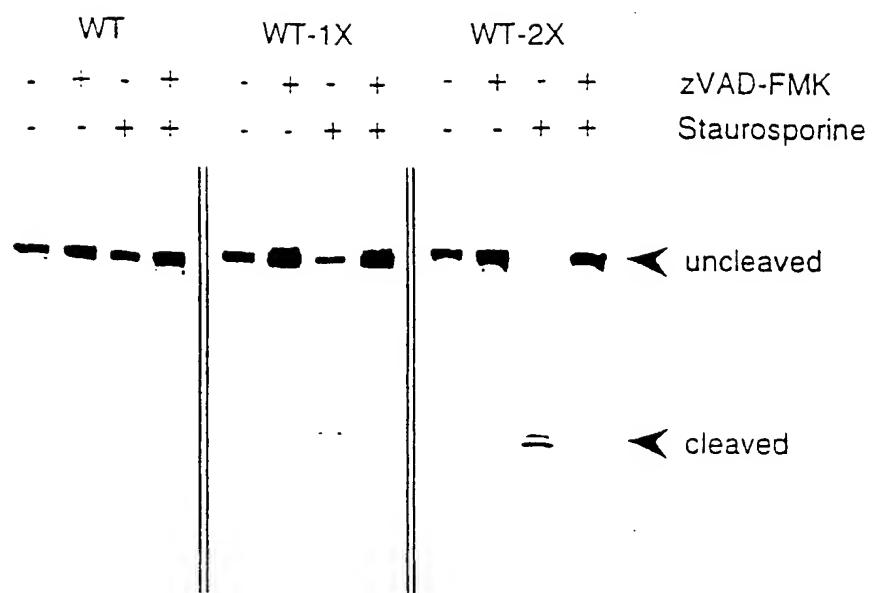


FIG.4A

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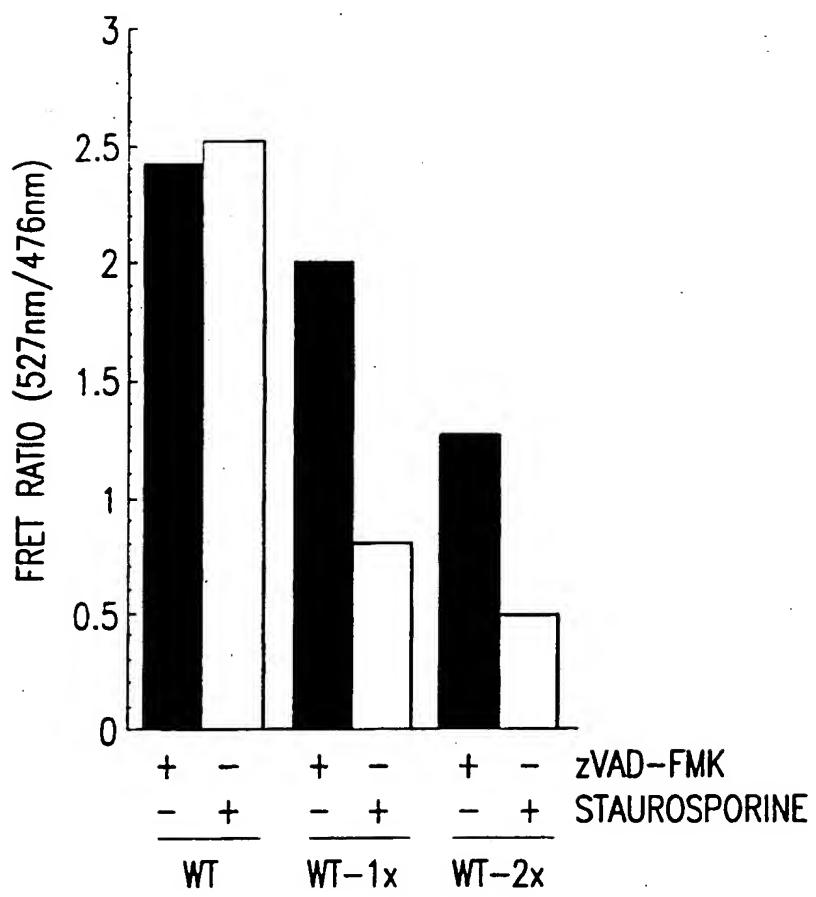
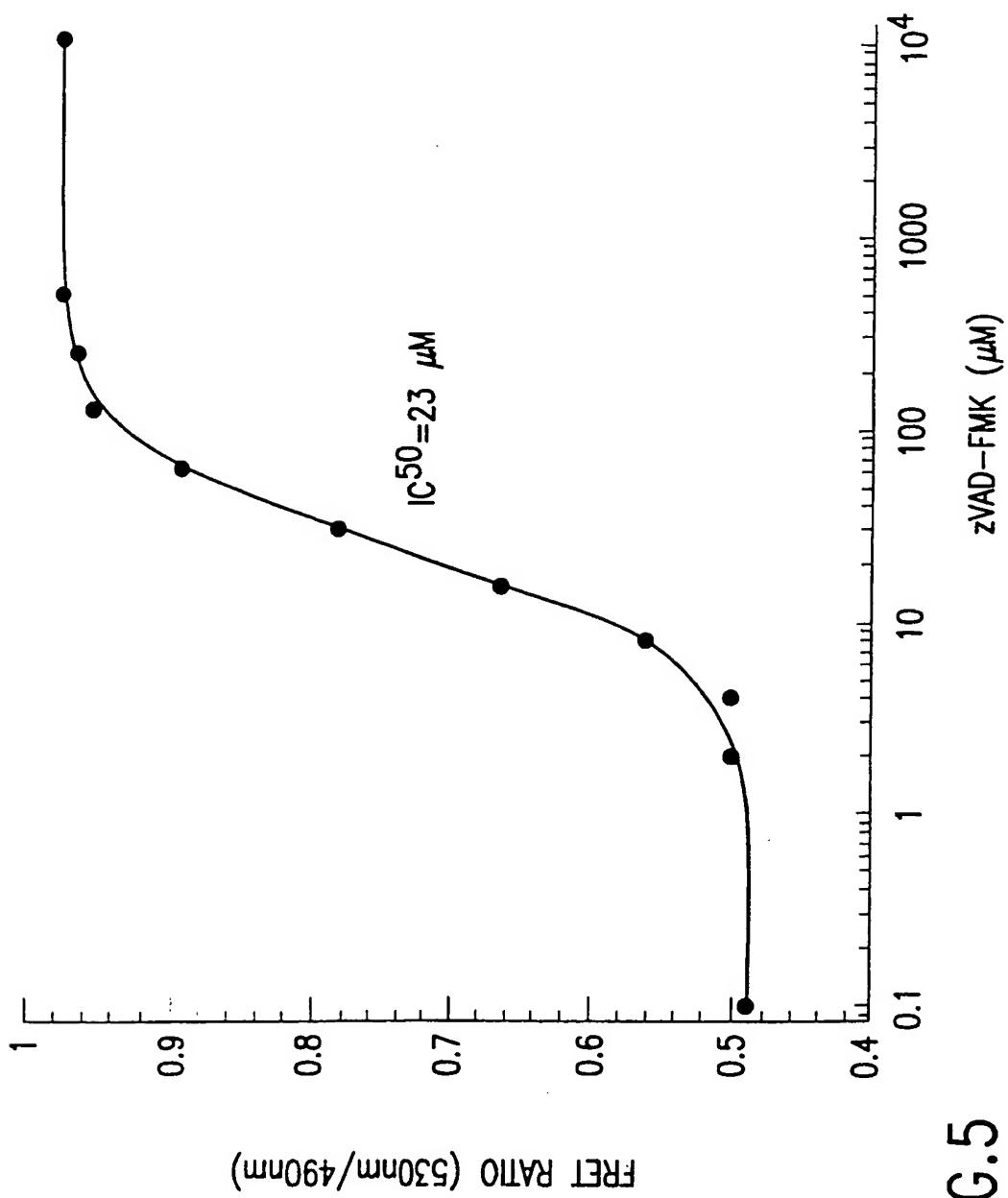


FIG. 4B

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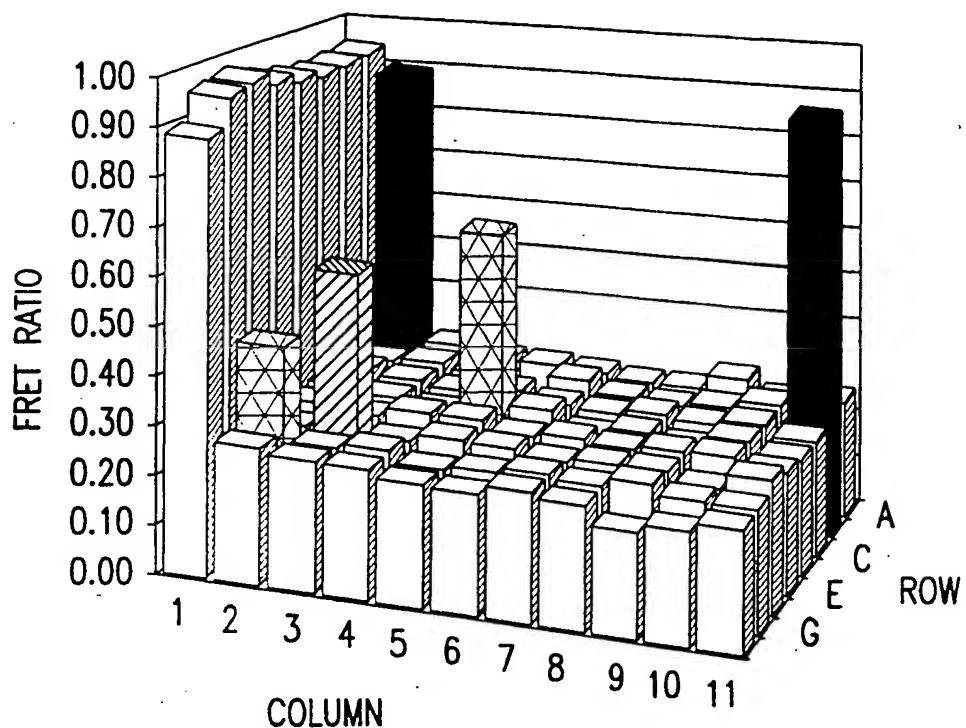


FIG. 6A

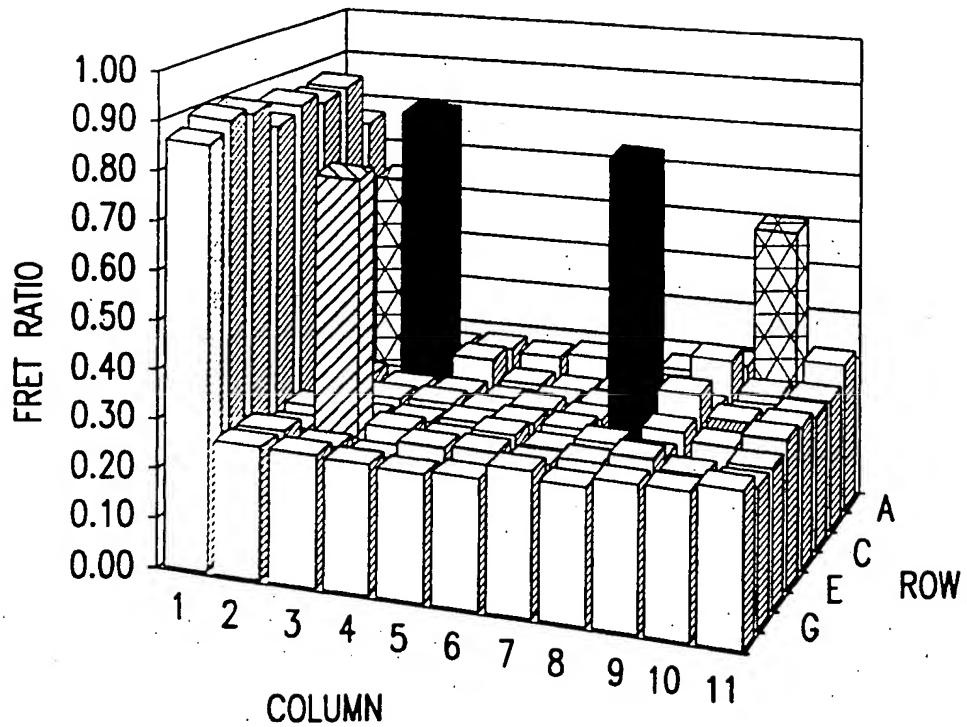


FIG. 6B

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1 tacacacgaa taaaagatcaa caaaggatgag taaaaggagaaa gaaacctttca ctggggtgt
61 cccaaatctt gttgaatttag atggcgatgt taatggggcaa aaatctctg tcgtggaga
121 ggglgaooggt gatgcaacat acggaaaaact taccctlaaa ttatatttca ctactggaa
181 gctacclgtt ccatggccaa cacttgcac tactttctct tatgggttca aatgttttc
241 aagataccca gatcatatiga aacagcaatga cttttcaag agtgcctatgc ccggaaaggta
301 tttacaggaa agaactataat tttacaaaga tgacgggaaac tocaagacac gtgtcaatgt
361 caaaggatggaa ggtgtatccc ttgttaatog aatcgatgtt aaaggatatttgg attttaaaga
421 agatggaaac attcttggac acaaaatggaa atacaactat aactcacata atgtatataat
481 catggcagac aaacccaaaga atggaaatcaa agttaatctc aaaaattttagac acaacatcaa
541 agatggaaac gttcaatattag cagaccatata tcaacaaaaat acttccaaatgg gcgtatggccc
601 ttttttttttta ccagacaacc attacctgtc cacacaatctt gcccatttcca aagatcccaa
661 cggaaaaggaaa gatcacatiga ttttttttttta gttttgttcaacaa gctgtgggtt ttttttttttta
721 catggatgaa ctatacaat aatgtccatg acttccaaat gacactaaatgg ttttttttttta
781 attatcttcaat ttttttttttta gttttgttcaacaa gctgtgggtt ttttttttttta
841 agatccatggaa aatttttatg aatcttctt ttttttttttta gatgttttttta aatgtggggctt ttttttttttta
901 ttttttttttta gttttgttcaacaa gctgtgggtt ttttttttttta (SEQ.ID.NO.:33)

```

FIG. 7A

1 MSKGEELFTG WPILVELDG DVNGQKF SVS GEGEGDATYC KLT LKF I CTT  
51 GKL PVPWFTL VTTFSYGVQC FS RYPD HMKQ H DFFKSAMPE G YVQERT I FY  
101 KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK MEYNYN SHNV  
151 YIMADKPKNG IKVNFKIRHN IKDGSVQLAD HYQQNTPIGD GPVLLPDNHY  
201 LSTQSALS K PNEKRDHMIL LEFVTAAGIT HGMDELYK (SEQ.ID.NO.:34)

FIG. 7B

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 00/00620

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N9/64 C07K14/435 C12Q1/37 G01N33/50  
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>°</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 28261 A (AURORA BIOSCIENCES CORP ;UNIV CALIFORNIA (US)) 7 August 1997 (1997-08-07) cited in the application claims 1,18,21,25-56 ---	1-13
Y	WO 99 18856 A (CYTOVIA INC) 22 April 1999 (1999-04-22) the whole document ---	1-13 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

### ° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "E" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

28 September 2000

Date of mailing of the international search report

13/10/2000

Name and mailing address of the ISA

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Authorized officer

Hornig, H

## INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 00/00620

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THORNBERRY N A ET AL: "A combinatorial approach defines specificities of members of the caspase Family and Granzyme B" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 29, 18 July 1997 (1997-07-18), pages 17907-17911, XP002145059 ISSN: 0021-9258 cited in the application the whole document ----	1-13
Y	XU XIANG ET AL: "Detection of programmed cell death using fluorescence energy transfer." NUCLEIC ACIDS RESEARCH, vol. 26, no. 8, 15 April 1998 (1998-04-15), pages 2034-2035, XP002148668 ISSN: 0305-1048 cited in the application the whole document ----	1-13
Y	MITRA R D ET AL: "FLUORESCENCE RESONANCE ENERGY TRANSFER BETWEEN BLUE-EMITTING AND RED-SHIFTED EXCITATION DERIVATIVES OF THE GREEN FLUORESCENT PROTEIN" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, GB, ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 173, no. 1, 1 July 1996 (1996-07-01), pages 13-17, XP002033688 ISSN: 0378-1119 cited in the application the whole document ----	1-13
Y	HEIM R ET AL: "ENGINEERING GREEN FLUORESCENT PROTEIN FOR IMPROVED BRIGHTNESS, LONGER WAVELENGTHS AND FLUORESCENCE RESONANCE ENERGY TRANSFER" CURRENT BIOLOGY, GB, CURRENT SCIENCE, , vol. 6, no. 2, 1 February 1996 (1996-02-01), pages 178-182, XP000676582 ISSN: 0960-9822 the whole document ----	1-13
		-/-

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 00/00620

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>ROMOSER V A ET AL: "DETECTION IN LIVING CELLS OF CA2+ -DEPENDENT CHANGES IN THE FLUORESCENCE EMISSION OF AN INDICATOR COMPOSED OF TWO GREEN FLUORESCENT PROTEIN VARIANTS LINKED BY A CLAMODULIN-BINDING SEQUENCE"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US,</p> <p>vol. 272, no. 20, 16 May 1997 (1997-05-16), pages 13270-13274, XP002058387</p> <p>ISSN: 0021-9258</p> <p>cited in the application the whole document</p> <p>-----</p>	1-13
A	<p>NICHOLSON D W ET AL: "Caspases: killer proteases"</p> <p>TIBS TRENDS IN BIOCHEMICAL SCIENCES, EN, ELSEVIER PUBLICATION, CAMBRIDGE,</p> <p>vol. 22, no. 8, 1 August 1997 (1997-08-01), pages 299-306, XP004085816</p> <p>ISSN: 0968-0004</p> <p>cited in the application the whole document</p> <p>-----</p>	

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/00620

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9728261	A 07-08-1997	AU	717873 B	06-04-2000
		AU	2250497 A	22-08-1997
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		EP	1026988 A	16-08-2000
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